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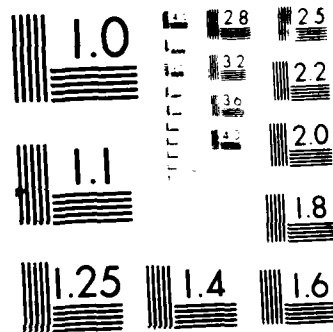
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**PURIFIED MOJAVE TOXIN INTERACTS
WITH $\text{Ca}^{++}\text{Mg}^{++}$ ATPASE IN RAT
SYNAPTIC MEMBRANES**

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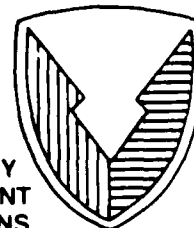
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PREFACE

The work described in this report was authorized under Contract No. DAAK-11-84-K-0003. This work was started in January 1986 and completed in April 1986. The experimental data are retained by the Division of Life Sciences, University of Texas at San Antonio.

In conducting the work described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Revision of the Guide for Laboratory Animals Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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PURIFIED MOJAVE TOXIN INTERACTS WITH $\text{Ca}^{++}\text{Mg}^{++}$ ATPASE IN RAT SYNAPTIC MEMBRANES

1. INTRODUCTION

The ($\text{Ca}^{++} + \text{Mg}^{++}$) ATPase of the synaptic membrane, as with the erythrocyte, is the enzyme possibly responsible for maintaining the concentration of Ca^{++} inside the respective cells at a much lower level than in the extracellular environment.¹ Calcium mediated channels produce changes in intracellular calcium in a voltage-dependent manner in a variety of excitable tissues.² The 1,4-dihydropyridine class of calcium antagonists bind saturably and reversibly with high affinity (K_d 1nM) to specific sites that appear to mediate the blockade of Ca^{++} flux through voltage-dependent calcium channels in a number of tissues.³

Mojave toxin is an acidic toxin isolated from the venom of Crotalus scutulatus scutulatus. Gel filtration studies indicate a molecular weight of approximately 22,000 daltons with an isoelectric point of 5.7.⁴ The toxin exists as a dimer with one monomer being very acidic and the second being basic and exhibiting phospholipase A activity.⁵ Polyacrylamide gel electrophoresis of sodium dodecylsulfate-treated toxin indicates that the monomeric molecular weight of the basic subunit is approximately 12,000. Early studies by Ginsborg and Warriner⁶ confirmed a presynaptic site of action of the toxin.

In this report, we describe in the presence of purified Mojave toxin: 1) a twofold increase in rat-brain synaptic membrane ($\text{Ca}^{++} + \text{Mg}^{++}$)-ATPase activity and 2) inhibition of the high-affinity calcium antagonist nitrendipine binding site. In the presence of the allosteric modulators, verapamil and diltiazem, nitrendipine binding was decreased greater than 90% in both instances, indicating that this toxin possibly interacts directly with several different sites. These observations suggest the possible usefulness of this purified snake toxin as a calcium channel complex probe.

2. MATERIALS AND METHODS

2.1 Materials.

Rabbit muscle adenosine triphosphate was obtained from Boeringer-Mannheim. HEPES, ouabain, sucrose, tris, dithiothreitol, phenylmethylsulfonylfluoride, polyethyleneglycol (molecular weight, 6000), cis-D-diltiazem, and (+/-) verapamil were obtained from Sigma Chemical Company. Male, Sprague-Dawley rats were obtained from Harlan Sprague, Inc., of Houston, Texas. (3H)-Nitrendipine was obtained from New England Nuclear. Unlabelled nitrendipine was a generous gift from Miles Pharmaceuticals. GF-B glass fiber filters were obtained from Fisher Scientific. 4-Bromophenacylbromide was purchased from Aldrich Chemical Company. Liquiscint scintillation fluid was obtained from National Diagnostics.

2.2 Preparation of Synaptosomes.

Animals, 150-175 gms, were killed by decapitation and brain tissue removed. Following removal and discarding of the brain stem, synaptosomes were prepared by the method of Hajos.⁷ Protein was determined by the method of Bradford⁸ using bovine serum albumin as a standard.

2.3 ATPase Assay.

Phosphate released by hydrolysis of ATP was monitored spectrophotometrically by the method of Lanzetta and co-workers.⁹ Briefly, all incubations contained in a final volume of 2 ml, 0.01 M Hepes buffer, pH 7.4, containing 50 μ M EGTA, 50 μ M ATP, and 0.3 μ M ouabain. Total ATPase +2 activity was determined in the presence of 100 μ M MgCl₂ and 2.52 μ M free Ca⁺⁺ ions. Magnesium-calcium dependent ATPase activity was determined by taking the difference between assays run in the presence of both Mg⁺⁺ and Ca⁺⁺ ions and those run only in the presence of Mg⁺⁺. All assays were conducted in triplicate with appropriate blanks. Assays evaluating the effects of 4-bromophenacyl-bromide on ATPase activity contained inhibitors at 1, 10, and 100 μ M final concentration.

2.4 Dihydropyridine Binding Assays.

Assays contained 100-125 μ g protein dissolved in 0.01 M tris, pH 8.2 buffer, containing 16% glycerol (v/v), and 1 nM (3H)-nitrendipine in a final volume of 1.0 ml. Nonspecific binding was determined by addition of 1 μ M unlabelled ligand (nitrendipine) or 1 μ M purified snake toxin. The molarity of snake toxin was calculated on the basis of a molecular weight of 22,000.⁴ Assays were terminated by addition of 3.0 ml ice-cold 0.1 M HEPES buffer, pH 7.4, containing 20% (v/v) polyethyleneglycol. Reaction mixtures were passed through GF-B glass fiber filters previously washed with 0.1 M HEPES buffer, pH 7.4, containing 8.5% polyethyleneglycol. Filters were immersed in 10 ml of liquiscint scintillation fluid and counted. All binding assays using soluble protein were carried out in identical fashion as described above, the exception being the addition of 0.5 ml bovine gamma globulin (10 mg/ml) as carrier protein immediately prior to termination. In order to determine the effects of two different allosteric calcium channel modulators of dihydropyridine binding, verapamil and diltiazem were added to each assay at a final concentration of 10 μ M and 30 μ M, respectively. Assays contained 10 μ g of purified snake toxin. In addition, the effects of 1, 10, and 100 μ M of 4-bromophenocylbromide on nitrendipine binding were determined. The specific activity of nitrendipine utilized in all binding assays was 312 dpm/fmole.

2.5 Purification of Mojave Toxin.

Mojave toxin was isolated and purified from C. scutulatus scutulatus venom by immunoaffinity chromatography.¹⁰

3. RESULTS

As shown in Figure 1, purified snake toxin stimulates (Ca⁺⁺ + Mg⁺⁺)-dependent ATPase approximately two-fold with no effect upon the Mg⁺⁺-dependent activity. Examination of the effects of increasing amounts of purified Mojave toxin on binding of the calcium channel blocker, nitrendipine is shown in Figure 2 where the addition of 10 μ g of toxin results in greater than 90% inhibition of nitrendipine binding. The addition of 20 μ g results in complete inhibition of binding. Binding data obtained in the presence and absence of synaptic membranes are summarized in Table 1. Synaptic membranes were observed to bind specifically 190.4 fmoles/mg protein (Experiments A and B). In the presence of unlabelled nitrendipine, 7.6 fmoles (3H)-nitrendipine per milligram

protein (approximately 3.4% total bound ligand) were observed to bind nonspecifically. As shown in Experiment C, purified Mojave toxin behaved in identical fashion as unlabelled ligand giving rise to 7.5 fmole (3H)-nitrendipine bound per milligram protein. The ligand bound in the presence of toxin appears to be nonspecifically associated as evidenced by the results of Experiment D. Only very small amounts of 3H-ligand (0.8 fmole/mg protein) were observed to bind to the toxin as indicated by Experiments E and F.

Since Mojave toxin has associated with it a phospholipase A2 activity, we investigated the possibility of using 4-bromophenacylbromide, a known inhibitor of phospholipase A2 activity,¹¹ to discern the possible effects of the purified toxin on synaptic membranes. As shown in Table 2, at low concentrations (1 μ M), the inhibitor had little effect on either (Ca++ + Mg++)-dependent ATPase activity or nitrendipine binding. In contrast, 100 μ M inhibitor resulted in significant inhibition of both parameters. The effects of two calcium antagonists that allosterically affect nitrendipine binding to the dihydropyridine recognition site are shown in Figure 3. Purified snake toxin (10 μ g) resulted in a decrease of specific 3H-ligand binding greater than 90%, labeled Control plus Toxin. Verapamil, in the absence of toxin, characteristically reduced binding by approximately 30% (X-X). In the presence of toxin and verapamil, nitrendipine binding was reduced greater than 90% (Verapamil plus Toxin in Figure 3). Diltiazem, a positive allosteric modulator, characteristically increased nitrendipine binding approximately 40% as compared to control (0-0). However, in the presence of toxin and diltiazem, nitrendipine binding was reduced approximately 90% (Diltiazem plus Toxin). The effects of the toxin plus the calcium antagonists at lower concentrations of nitrendipine were too small to measure reliably.

4. DISCUSSION

Movement of Ca++ across the plasma membrane of the presynaptic portion of nerve cell is crucial to transmission of the nerve impulse across the synaptic cleft. Depolarization of the presynaptic membrane is believed to open channels allowing extracellular Ca++ and raise the Ca++ concentration inside. Intracellular free Ca++ must be decreased to low concentrations by sequestration or pumping back out across the membrane. Data presented here indicate that purified Mojave toxin effectively stimulates (Ca++ + Mg++)-dependent ATPase activity twofold while inhibiting binding of the channel antagonist, nitrendipine. It is of interest that the (Ca++ + Mg++)-dependent ATPase activity has previously been shown to be stimulated by the very acidic protein, calmodulin.^{12,13} Since the active toxin consists of an acidic polypeptide tightly associated with a basic polypeptide exhibiting phospholipase activity, it is possible that the acidic moiety mimics to some extent the calmodulin molecule.

An alternative explanation entails removal of specific fatty acid moieties from surrounding phospholipids by the associated phospholipase activity, giving rise to changes in the immediate lipid environment of the ATPase. As indicated in Table 2, the known phospholipase A2 inhibitor 4-bromophenacylbromide was observed to inhibit significantly both ATPase and Ca++ channel antagonist binding at a concentration (100 μ M) previously shown to be effective in inhibiting 25-50 μ g purified cobra venom phospholipase.¹¹ Thus, the usefulness of this inhibitor in discriminating the possible role of the endogenous phospholipase activity is significantly diminished. The toxin's altering of key channel phospholipid components via the endogenous A2 lipase activity cannot be ruled out at this time. This is an important consideration especially in light

of Glossman and Ferry's¹⁴ observation that solubilized high-affinity binding sites for nimodipine, a dihydropyridine calcium channel blocker, are sensitive to phospholipases A and C. However, solubilization of the dihydropyridine binding sites might have rendered the channel complex more susceptible to phospholipase activity than that of the native untreated synaptic membranes used in this study. Inclusion of an excess of phospholipase A2 substrate (4 mM dipalmitoylphosphatidylcholine) in the binding assay resulted in significant (greater than 75%) inhibition of both control nitrendipine binding and (Ca++ + Mg++)-dependent ATPase activity (data not shown). Examination of the effect of snake toxin on the binding of verapamil and diltiazem to a second receptor site that allosterically regulates the binding of nitrendipine indicated total abolition of the interaction of both diltiazem and verapamil. This is of interest because binding studies with radioactive derivatives have shown that dihydropyridine derivatives recognize a binding site distinct from that of verapamil and diltiazem.^{15,16}

5. CONCLUSIONS

With the possible exception of maitotoxin,¹⁷ no potent naturally occurring toxin for the Ca++ channels is known. A variety of organic calcium channel blockers exists. Data presented here indicate that Mojave toxin affects both (Ca++ + Mg++)-ATPase activity and binding of nitrendipine to the high-affinity Ca++ channel receptor. Thus, purified snake toxin is a potentially useful probe for study of the Ca++ channel complex. However, these results should not be interpreted as being specific only to the Ca++ channel since the possible blocking of other channels was not determined in this study.

Table 1. The Effects of Purified Mojave Toxin on Dihydropyridine Binding

	fmoles bound/mg protein
A. Synaptic Membranes + 3H-ligand	198.0
B. Synaptic Membranes + 3H-ligand + Unlabelled Ligand	7.6
C. Toxin + Synaptic Membranes + 3H-ligand	7.5
D. Toxin + Synaptic Membranes + 3H-ligand + Unlabelled Ligand	7.6
E. Toxin + 3H-ligand	3.8
F. Toxin + 3H-ligand + Unlabelled Ligand	3.0

Table 2. The Effects of 4-Bromophenacylbromide
Treatment on (Ca⁺⁺ + Mg⁺⁺) ATPase and
Nitrendipine Binding

4-Bromophenacyl- bromide (μ M)	% Control ATPase	% Control Nitren- dipine Binding
1	78	100.0
10	54	80.5
100	25	30.0

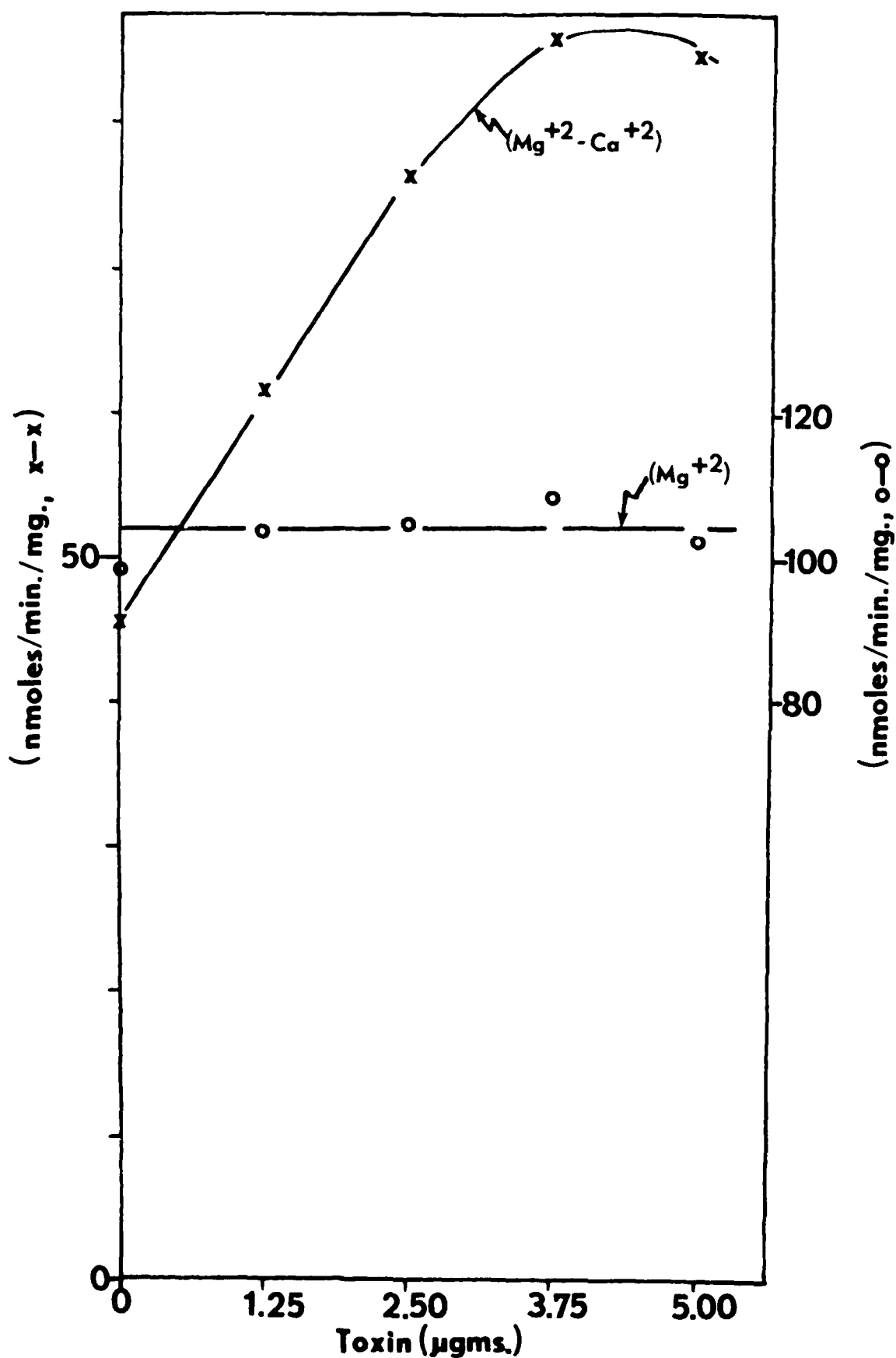


Figure 1. Effects of Purified Mojave Toxin on Mg^{++} - and $(\text{Ca}^{++}\text{Mg}^{++})$ -Dependent Rat Brain Synaptic Membrane ATPase.

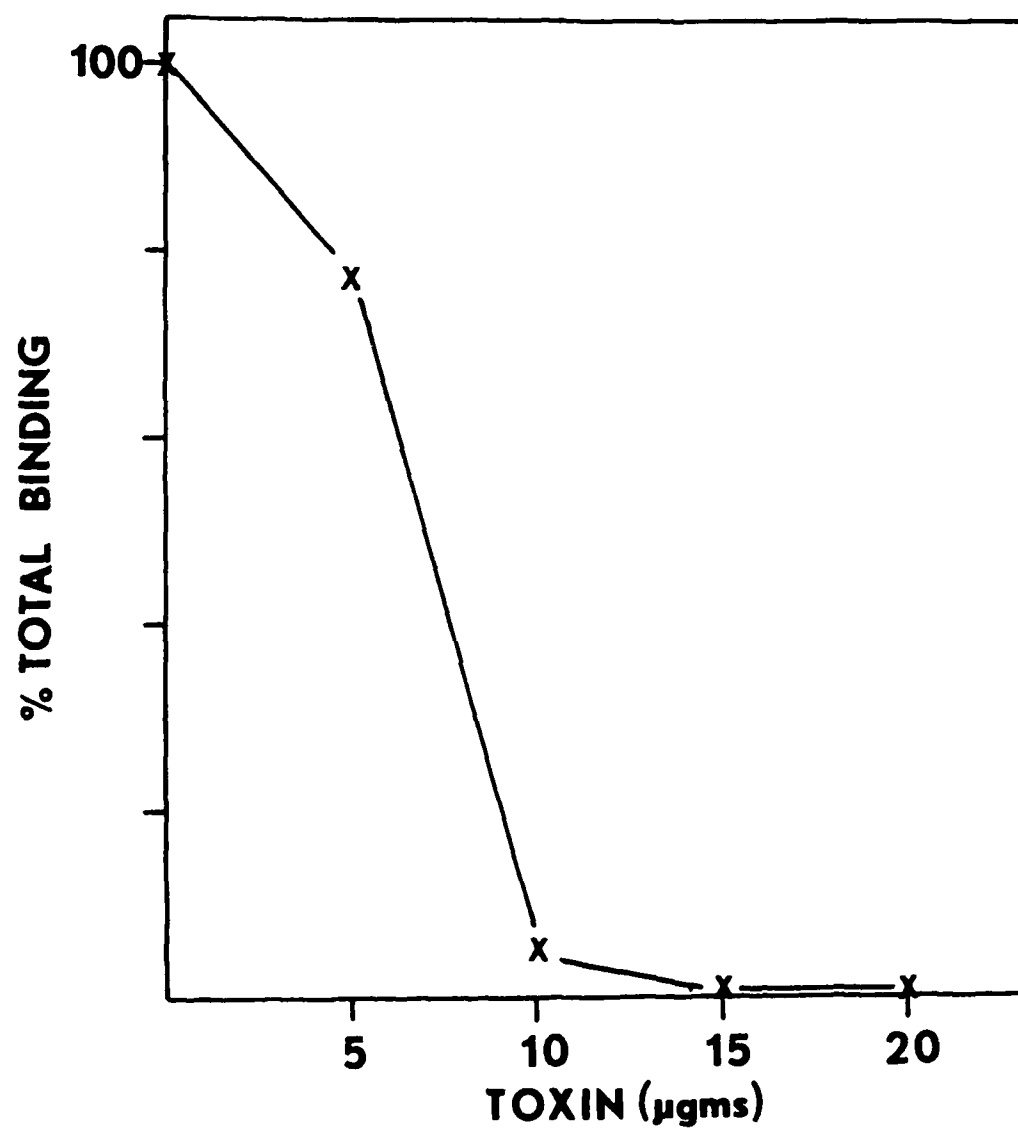


Figure 2. Effects of Purified Mojave Toxin on Rat Brain Synaptic Membrane Nitrendipine Binding.

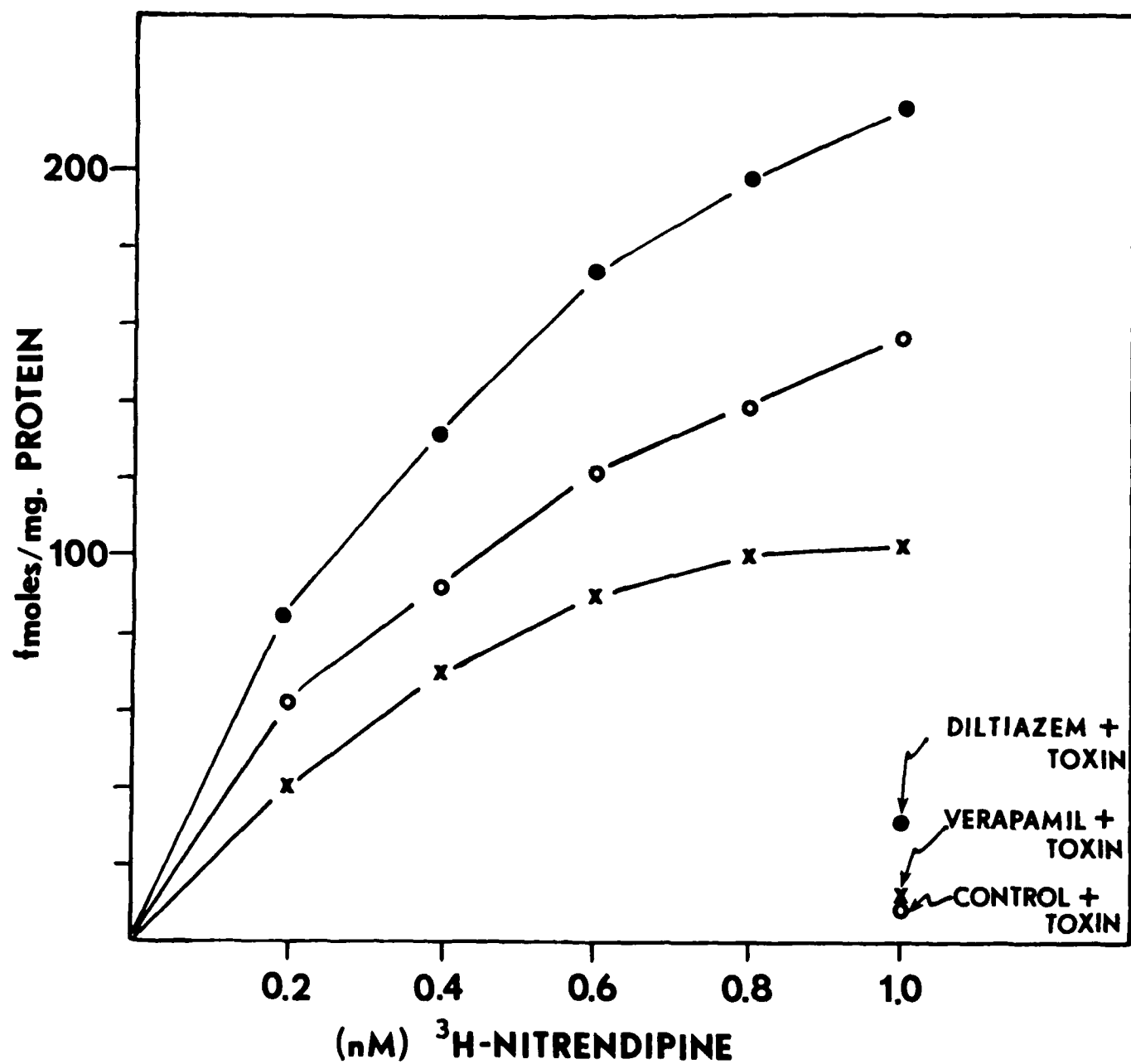


Figure 3. Effects of Purified Mojave Toxin on the Interaction of Diltiazem and Verapamil with the High-Affinity Nitrendipine Receptor.

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